CONTRIBUTIONS TO OUR KNOWLEDGE OF SOIL-FERTILITY,

xii. The Action of Toluene upon the Soil-Protozoa.

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In their experiments upon the partial sterilisation of soils, Russell and Hutchinson, as a rule, employed toluene in the proportion of from 0·5 % to 1 % of the soil, leaving it to act for about 30 hours, and then allowing it to evaporate completely.* They found that 1 % of toluene entirely suppressed the detrimental factor, which limits the growth of bacteria, when the disinfectant was allowed to act for two days.† In richer soils, however, the toluene often failed to kill all the protozoa, just as it failed to destroy nitrifying organisms, and to cure soil-sickness. The failure was traced to the low solubility of toluene, and its consequent inability to penetrate any but the smallest particles of soil, in the presence of much moisture or organic matter.‡ Regarding certain discrepancies between laboratory and pot-experiments, Russell and Petherbridge said that their experiments showed that toluene acted best in finely sifted, fairly dry soils, and lost much of its effectiveness in rich soils, when too much moisture was present or the soil-particles were too coarse.§ Yet, in their conclusions upon their work with sickness in glass-house-soils, which, I submit, must be classed as "rich" soils, they said that the factor, detrimental to bacteria, resembles in every way that present in ordinary arable soil. It was put out of action by antiseptics, and, in all respects, its properties agreed with those of the protozoa.‖

In considering these various findings, one is driven to the conclusion that, as protozoa cannot be trusted to be absent in

* Journ. Agric. Science, v., p.157.

† *Op. cit.*, p.171. ‡ *Op. cit.*, p.190. § *Op. cit.*, p.107. ‖ *Op. cit.*, p.111.

rich soils, which have been treated with toluene, all work done upon such soils cannot be taken as indicating the inactivity or otherwise of the protozoa. The bulk of the work was done, as they say, with 1 % of toluene, and it was taken, generally, that the protozoa had been eliminated, yet their latest work contains many footnotes to the effect that the protozoa had not been destroyed.

There is so much claimed for the protozoa by Russell and his co-workers, and, at the same time, there are so many points wherein they safeguard themselves, while pushing forward the protozoal hypothesis, that it appeared to be opportune to examine the limits of the action of the volatile disinfectant, toluene. This was all the more desirable, as I had already found that even 10 % of toluene did not kill off all the ciliates in soil.

In the experimental work embodied in this paper, the general procedure consisted in sifting the soil through sieves varying from 12, for damp soils, to 30 meshes to the inch, for dry soils. Weighed portions were put into bottles, treated with varying amounts of toluene (Kahlbaum), and thoroughly stirred with a sterile wire. Disinfection was allowed to act for two days. The soils were then spread on paper, and returned to their bottles when the toluene had evaporated. About five grams of each portion of the soil were put into inch test-tubes, containing 10 c.c. of culture-fluid, and these were incubated at 22°. At convenient intervals, generally 3, 5, 7, and 10 days, a small quantity of the fluid was carefully pipetted from the surface-edge of the liquid, while the point of the capillary pipette was rubbed against the glass wall of the tube. This was done with the intention of dislodging the amœbæ, and allowing them to be sucked up in the pipette. The teat was then pressed, and the drop blown upon a glass-slide, which was dusted with fine sand, and a cover glass was placed over the drop. When the amœbæ had been given time to settle and attach themselves to the surface of the slide, the film was examined.

Many culture-fluids were tried. At first, an infusion of French beans was used, but an improvement was found in dilute nitrate bouillon, containing one part of ordinary nitrate bouillon to seven

parts of water. The addition of 0·1 % of dextrose greatly helped the growth of the protozoa, but, noting that Killer had found Giltay's solution to be one of the best for the purpose, it was tested and found to be eminently suitable. The weakening of the Giltay's solution did not accelerate the growth of the protozoa, neither did the replacement of the potassium nitrate by similar weights of either asparagin, peptone, ammonium sulphate or chloride, urea, or of meat-extract. Finally, 1% hay-infusion with 0·1 % of nitrate was shown to be best for the growth of ciliates, and Giltay's solution for the growth of amœbæ.

With the idea of obtaining a maximum growth of protozoa, a poor alluvial soil* from the Hawkesbury Agricultural College was air-dried, sifted, moistened, and spread out in a large photographic developing dish. It was maintained in the moist condition for three weeks. Portions of the soil, containing 10 % of moisture, were treated with varying amounts of toluene. The remainder was air-dried, and also treated with toluene.

Moist soil	Ciliates survived 1·5 %, killed by 2 %.
	Amœbæ survived 2 %.
Air-dried soil	Ciliates and amœbæ survived 20 %.

The sandy soil of the Society's garden contained 12 % of moisture. It was treated with toluene at once, and then air-dried.

Moist soil	Ciliates survived 1 %, killed by 2 %.
	Amœbæ survived 2 %, killed by 5 %.
Air-dried soil	Ciliates and amœbæ survived 20 %.

Control-experiments confirmed these findings.

During the experiments, which were comparatively numerous, it was noted that the ciliates were less in number, and that the flora was more varied in the untreated, than in the toluened

* The protozoa, noted in cultures from this soil, were *Colpoda cucullus*, *Vorticella*, *Paramœcium*, *Peridium tripos*, *Trachelocerca viride*, *Cercomonas*, *Chætomonas*, *Dileptus*, *Trepomonas agilis*, *Amœba lobosa*, *Amœba diffluens*.

soils. The volatile disinfectant favoured the development of an active, though restricted, fauna, and a flora consisting largely of slime-forming bacteria. The control-tests had a very diverse flora and fauna, while the toluened tests grew a very numerous crop, consisting almost entirely of colpodæ, amœbæ, and small monads, and the suspensions were covered with a thick, gelatinous film of bacteria. In Giltay's solution, the controls were always of a paler colour than the treated tests. The flora had undoubtedly suffered a considerable diminution in its species, and the idea appeared feasible, that, possibly, the reason for the non-appearance of the protozoa in the toluened moist soils, might be due to the absence of certain bacteria, killed off by the toluene.

In order to test the matter, a raw garden-soil was shaken up in Giltay's solution, and the supernatant liquid was centrifugalised. Minute drops were taken, and examined microscopically for protozoa. Those free from all suspicion of protozoa, were taken up in capillary tubes and dropped into Giltay's solution. The growth consisted of bacteria only. Drops of this subculture were introduced into tubes containing Giltay's solution and portions of treated soil. Examination of these, from time to time, showed that they were no better, so far as the growth of protozoa was concerned, than tests without the addition of the bacteria. The idea that the bacteria might have an influence upon the development of the fauna in treated soils, therefore, was not substantiated.

In one medium, namely, dilute dextrose nitrate bouillon, an observation was made, which appeared to explain the inability of ciliates to grow in the tests made with the alluvial soil. Other media containing easily reducible organic matter, such as hay-infusion, showed the same phenomenon. The soil is of a pale smoke-colour, and it was noted that the medium remained normal, when ciliates were present, but, in their absence, a dark-coloured, filmy growth formed in the dilute bouillon. This settled on the sedimented soil, the upper layers of which were distinctly black. The dark-coloured film consisted of bacteria and soil-particles, containing black fragments, which became

blue upon treatment with acetic acid and potassium ferricyanide. Sulphide of iron was thus indicated. The presence of sulphide and the absence of ciliates, and vice-versâ, point to the possibility of the sulphur-oxidising bacteria having been destroyed by the treatment with toluene. The fact was recalled that, in some of the tests, the amœbæ moved sluggishly, and it appeared probable that they had been partly poisoned by traces of sulphuretted hydrogen in the culture-fluids.

The following experiment was made to confirm the idea. Garden-soil was suspended in Giltay's solution in a conical flask, containing a test-tube holding potassium sulphide. A few drops of dilute sulphuric acid were added to the sulphide, and the flask was corked. Five days later, amœbæ and monads were detected, but no ciliates were found. The living protozoa moved slowly, and were undoubtedly unhealthy. In another experiment, a stream of sulphuretted hydrogen was passed through two bottles, one containing a damp, the other a dry soil. After five minutes, the bottles were corked. Next day, the soils were spread out and aired. The dry soil, when sown in Giltay's solution, gave rise to a mixed fauna; the damp soil was free from protozoa.

It is clear that, if the conditions are such that a reduction of sulphate is possible, as, for example, in soils containing much organic matter, there is the possibility that the action of a volatile disinfectant, by destroying some of the groups of the sulphur-oxidising bacteria, may indirectly affect the growth of the protozoa, and especially of the ciliates. This, however, is not always the reason for the non-appearance of ciliates in cultures from toluened damp soils, as will be shown subsequently.

In view of the fact that toluene had been found to have no effect upon protozoa in air-dried soil, so far as certain members of each group were concerned, it was thought advisable to confirm the matter by examining a few more soils. Accordingly, six fresh soils were obtained from the Hawkesbury Agricultural College, through the kindness of the Principal, Mr. H. W. Potts. The weather had been very dry for the preceding four months, and the soils reached the laboratory in the air-dry condition. The moisture ranged from 0·5 % in a gravelly soil (W.H.C. = 16),

to 2·3 % in a chocolate loam (W.H.C. = 36). Treatment with varying quantities of toluene showed that all percentages, up to twenty, failed to destroy the protozoa, taking *Colpoda cucullus* as being typical of the ciliates, *Cercomonas* or *Trepomonas* as representing the flagellates, and the usual *Amœba limax* or *lobosa* for the amœbæ.

Gravelly Soil.—W.H.C., 16.

Moisture-percentage.	9·3	5·2	4·0	3·5	2·0	0·3
No toluene	C.A.M.	C.A.M.	C.A.M.	C.A.M.	C.A.M.	C.A.M.
Toluene, 1%...	none	none	none	M.	M.	C.A.M.
Toluene, 2%...	none	none	A.	M.	A.	C.A.M.
Toluene, 5%...	none	none	A.	none	none	C.A.M.
Toluene, 10%	none	A.M.	M.	M.	none	C.A.M.
Toluene, 20%	none	none	M.	A.M.	M.	C.A.M.

Chocolate Loam.—W.H.C., 36.

Moisture-percentage.	17·6	13·8	13·5	11·3	9·1	6·9	3·0	2·7
No toluene ...	C.A.M.	C.A.M.	C.A.M.	C.A.M.	C.A.M.	C.A.M.	C.A.M.	C.A.M.
Toluene, 1% ..	M.	A.M.	—	M.	A.M.	A.M.	C.A.M.	C.A.M.
Toluene, 2% ...	none	A.	A.	A.M.	A.	A.M.	C.A.M.	C.A.M.
Toluene, 5% ...	none	A.	A.	A.M.	A.	A.M.	A.M.	C.A.M.
Toluene, 10%...	none	none	A.M.	M.	A.	A.M.	A.M.	C.A.M.
Toluene, 20%...	none	none	A.M.	A.M.	none	A.M.	A.M.	C.A.M.

C. = ciliates; A. = amœbæ; M. = monads or flagellates.

The two extreme soils, one with a W.H.C. of 16, and the other of 36, were put into jars, and water was added to over half the water-holding capacity. They were kept for two months at the

air-temperature, the water being replaced from time to time as it evaporated. After this time, they were allowed to dry slowly, while portions were taken at intervals and treated with toluene. Giltay's solution was used for the cultivation.

The experiments show that there is considerable irregularity, either in the effect of the disinfectant or in the capability of growth after treatment. It appears, however, that toluene has little disinfecting action, when the moisture-content is lower than from one-tenth to one-twentieth of the water-holding capacity of the soil, and that when soils are quite moist, amœbæ and flagellates may not be affected to any great extent. It is possible that, had nitrate hay-infusion been used instead of Giltay's solution, the ratios would have been narrower.

It appears to make no difference, whether the water is originally present in the soil, or is added at the time of toluening. A garden-soil with 6·2 % of moisture was allowed to dry slowly in the air, while portions were taken from time to time, and treated with 1 % of toluene. When air-dried, the soil was moistened with varying quantities of water, and treated straightway with toluene.

Garden-soil (W.H.C.=40).					Field-soil (W.H.C.=25).			
	Soil slowly dried.		Moisture added.		Moisture added.			
Moisture %	Giltay.	Nitrate hay-infusion.	Moisture %	Giltay.	Moisture %	Giltay.	Nitrate hay-infusion.	Nitrification, six weeks.
6·2	A.M.	M.	5·9	A.M.	3·0	A.M.	none	none
4·4	A.M.	M.	4·4	A.M.	2·5	A.M.	A.	none
4·1	A.M.	M.	3·9	A.M.	2·0	A.M.	A.M.	none
2·9	A.M.	C. M.	3·9	M.	1·5	A.M.	A.M.	none
1·5	A.M.	C.A.M.	2·0	A.M.	1·0	A.M.	C.A.M.	none
1·4	C.A.M.	C.A.M.	1·4	C.A M.	0·5	C.A.M.	C.A.M.	active

These experiments show that, if the moisture falls below one-twentieth of the water-holding capacity, one per cent. of toluene

does not destroy the ciliates completely, as tested by nitrate hay-infusion, or one-fortieth as tested by Giltay's solution. Incidentally, they also show that nitrate hay-infusion is better suited for the growth of ciliates, and Giltay's solution for the growth of amœbæ.

The influence of the soil-moisture upon the action of the disinfectant, points to the ciliates either existing in the moist soil in the motile state, or, if encysted, to the cyst-membrane being more pervious to the combined action of moisture and toluene. The age of the cyst may be a controlling factor. Recently encysted ciliates and amœbæ are delicate and colourless, while the older ones are more or less brown and look undoubtedly stronger. One can imagine that the destruction of the delicate cyst may be an easy matter compared with the stronger.

In the belief that a knowledge of the effect of heat might throw some information upon the action of the volatile disinfectants, a series of examinations was made upon the appearance and the activities of protozoa after they had been heated at different temperatures. Cultures of protozoa in Giltay's solution were taken up in capillary pipettes, and after the end had been sealed, they were heated in water for ten minutes at different temperatures. The pipette was then cooled, the point broken off, and the suspension blown upon a slide and examined.

Paramœcium was either still or moved about slowly after having been heated at 39° or 40°. At 41°, it became quite still, with the cilia moving slowly. At 42°, the organisms were completely broken up into masses of débris. *Colpoda cucullus* behaved somewhat differently, according to the size of the cells. At 39°, some of the smaller or younger organisms were slowly motile, others had stopped moving about the field, but the cilia still vibrated; others were more or less spherical, with an extruding slime-drop. At 40°, they were all non-motile, mostly rounded, with a lateral slime-drop. This extrusion of slime occurs during rapid encystment. Heated at 41°, they were all rounded, and some appeared to have burst. The nuclei of the rounded cells stained with methylene blue, the rest of the cell did not take up the colour, from which it is inferred that the

cells were not dead but were encysting. The older cells at 41° or 42° moved about, but, at 43°, they had come to rest, and a few showed the cilia moving slowly, while others were rounded and entire or had apparently burst. At 44°, they were spherical or slightly ovoid, and were apparently encysting.

The amœbæ began to be affected at 43°, when their motility ceased. At 44°, they were rounded or spherical, some with the pulsating vacuole active, others with it still. That they were not dead, was shown by their refusing to take up methylene blue.

The flagellates became non-motile and irregularly shaped at 39°, and did not take the stain. Tabulating these observations, we have: —

	Immobile.	Rounded.	Destroyed.
Paramæcium	41°	—	42°
Colpodæ, mature	43°	44°	—
Colpodæ, immature	40°	41°	—
Amœbæ	43°	45°	—
Flagellates	39°	40°	—

Omitting the flagellates, which appear irregular, the action of heat upon the protozoa is very similar to the action of the volatile disinfectants. Paramæcium is quickly destroyed, while the amœbæ are less affected than Colpoda. Still, there should be a greater difference between the amœbæ and the mature colpodæ.

The motile colpodæ and amœbæ, after becoming immobilised and rounded at 44° and 45°, behaved to stains as if they were still alive and encysting, but they really had become so altered that subsequent growth in nutrient solutions did not occur. This was shown by suspensions, which contained motile forms only, becoming sterilised after being heated for ten minutes at 45° (amœbæ), and 46° (colpodæ).

The lethal temperature of the protozoa, as occurring in the soil, is always higher than the motile forms, on account of the presence of cysts. Work upon such encysted forms has been done by others, but a few tests are given, chiefly to show the influence of the culture-fluid.

LETHAL TEMPERATURES OF SOIL-PROTOZOA.

	Culture-fluid.	Ciliates.	Amœbæ.	Flagellates.
Moist soil, No.2 ...	Giltay	under 54°	63°	under 54°
Moist soil, No.1 ..	Giltay	56°	over 62°	over 62°
Air-dried soil, No.4	Giltay	54°	70°	58°
Air-dried soil, No.4	1% hay-infusion	50°	64°	58°

The low lethal temperature in hay-infusion, as compared with Giltay's solution, was traced to the presence of sulphuretted hydrogen. On the twelfth day of cultivation, a slight deposit of ferrous sulphide was noted lying on the surface of the soil, in the tube which had been heated at 50°, while, at 52° and all higher temperatures, the deposit of the black sulphide was pronounced. The sulphide films were not seen in Giltay's solution.

The development of sulphuretted hydrogen in the hay-infusion indicates that this medium is not suitable for demonstrating the effect of heat upon the protozoa. It is the medium which has been used by many investigators for work connected with the action of heat and volatile disinfectants upon the protozoa. As it is unsuited for showing the action of heat, it is possible that it may also be unsuitable for growing the fauna, that survive in soils, treated with volatile antiseptics. A test was made, therefore, with two light-coloured soils. But for the use of such pale-coloured soils, the films of ferrous sulphide would probably never have been detected.

A light-coloured soil, with a W.H.C. of 25, contained 7·5 % of moisture when toluened. After treatment, it was sown in tubes of hay-infusion and of Giltay's solution, and the suspensions were examined on the 2nd, 7th, and 11th days.

	1 % Hay-infusion.		Giltay's solution.	
	Protozoa.	Sulphide-formation.	Protozoa.	Sulphide-formation.
Control	C.A.M.	none	C.A.M.	none
Toluene, 0·5 % ...	A.	slight	A.M.	none
Toluene, 0·75 % to 20 %	none	pronounced	A.M.	none

Another soil with 0·5 % of moisture was treated at the same time, and sown in hay-infusion. Ciliates, amœbæ, and monads were found in all the tests, from none up to 20 %. There was only a suspicion of sulphide in all the tubes, with the exception of the control, 3 %, and 4 %, which were free from any trace.

The same light-coloured soil, with 11·6 % of moisture, was toluened, and subsequently sown in 1 % hay-infusion, with and without the addition of 0·1 % potassium nitrate.

	1 % Hay-infusion.		Hay-infusion with nitrate.	
	Protozoa.	Sulphide-formation.	Protozoa.	Sulphide-formation.
Control	C.A.M.	none	C.A.M.	none
Toluene, 0·5 % to 20 %...	A.M.	pronounced	A.M.	none

In these experiments, we see that, after toluening a damp soil and adding it to 1 % hay-infusion, there is a formation of sulphuretted hydrogen similar to what was obtained in dilute dextrose nitrate bouillon.

The presence of potassium nitrate in Giltay's solution and in the nitrate hay-infusion prevented the formation of ferrous sulphide, but it did not accelerate the development of the ciliates. The ciliates were destroyed by the toluene, and it was immaterial whether sulphuretted hydrogen was formed in the culture-medium or not.

Sulphides were found in media containing easily reducible organic matter, such as 1 % hay-infusion and dilute dextrose nitrate bouillon, but not in Giltay's solution or in hay-infusion with nitrate, even although the latter never became acid to litmus-paper.

Regarding the action of toluene upon the bacteria, there is indicated, in the formation of the sulphide, the probability that certain oxidising organisms in the wet soils are, by the treatment, either destroyed or overwhelmed in numbers by the surviving reducing bacteria. In dry soil, this does not occur.

Conditions which cause the apparent destruction of the sulphur-oxidising bacteria also cause the destruction of the ciliates. This is much akin to the conclusion of Russell and his colleagues, who say that conditions which preclude the presence of the nitrifying bacteria, also preclude the presence of a mixed fauna. While this does not appear to hold for the nitrifying bacteria (see p.845) of dry soils, one has to bear in mind that experiments with the protozoa are always more or less variable.